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Occurrence of a Tyr393→Asn (Y393N) Mutation in the E1α Gene of the Branched-Chain α-Keto Acid Dehydrogenase Complex in Maple Syrup Urine Disease Patients from a Mennonite Population

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Summary

Maple syrup urine disease (MSUD) is caused by a deficiency in the mitochondrial branched-chain α-keto acid dehydrogenase complex. The incidence of MSUD in the Philadelphia Mennonites is 1/176 births resulting from consanguinity. In this study, we amplified cDNAs for the decarboxylase E1α subunit of the branched-chain α-keto acid dehydrogenase complex from a classical MSUD patient and from an obligatory heterozygote of a Mennonite family by the PCR. Sequencing of the amplified cDNAs disclosed at codon 393 of the mature E1α polypeptide a base substitution changing a tyrosine (encoded by TAC) to an asparagine residue (encoded by AAC), which is designated Y393N. A segment of the E1α gene containing the 5′ portion of exon 9 was amplified. Probing of the amplified genomic DNA with allele-specific oligonucleotide probes showed that the mutation in the E1α gene was homozygous in six Mennonites affected with classical MSUD and was present in heterozygous carriers. The identification of the MSUD mutation in the Philadelphia Mennonites will facilitate diagnosis and carrier detection for this population.

Introduction

Maple syrup urine disease (MSUD), or branched-chain ketonuria, is an autosomal recessively inherited deficiency in the mitochondrial branched-chain α-keto acid dehydrogenase (BCKAD) complex (Danner and Elsas 1989). The multienzyme complex degrades the α-keto acids derived from the three branched-chain amino acids leucine, isoleucine, and valine. The mammalian BCKAD complex is a macromolecule consisting of three catalytic components: i.e., a decarboxylase (E1) composed of two α (M_r 47,000) and two β (M_r 37,000) subunits, a transacylase (E2) core consisting of 24 identical lipoate-bearing subunits (M_r 46,500), and a dehydrogenase (E3) that exists as a homodimer (monomer M_r 52,000) and is shared with the pyruvate and α-ketoglutarate dehydrogenase complexes (Yeaman 1989). In addition, the mammalian BCKAD complex contains two regulatory enzymes, a specific kinase and a specific phosphatase that control the activity of the enzyme complex through a phosphorylation/dephosphorylation cycle (Yeaman 1989).

MSUD is genetically heterogeneous, as the BCKAD complex is encoded by at least six structural genes, and deficiencies in various protein subunits have been shown (Danner and Elsas 1989; Fisher et al. 1989). While the prevalence in the general population is estimated to be between 1/84,000 and 1/214,000 (Danner and Elsas 1989), the incidence of the classical form of MSUD in the Philadelphia Mennonites is much higher, approximately 1/176 live births (Marshall and DiGeorge 1981). In the present study, we investigated the MSUD mutation in classical patients, obligatory heterozygotes, and siblings from a Mennonite family by sequencing E1α cDNAs amplified by the PCR. This led to the finding that a common mutation is present in the E1α gene of Philadelphia Mennonites.

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Material and Methods

Cell Lines

Fibroblasts or lymphoblasts (subjects LMK and MaK) from members of a Mennonite (K) family (parents PaK and MaK, classical MSUD patients PK and LMK, and unaffected siblings EK and LK) and from two other Mennonite classical MSUD patients (AF and MZ) were provided by Drs. A. DiGeorge and Iraj Rezvani (St. Christopher's Hospital for Children, Philadelphia). Fibroblasts from another Mennonite classical MSUD patient (NN) and from a variant MSUD patient of non-Mennonite ancestry (M Ko) were provided by Dr. Marc Yudkoff (The Children's Hospital of Philadelphia, Philadelphia). Lymphoblasts from a Mennonite classical MSUD patient (GM 1655) were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). Fibroblasts from Misk., a non-Mennonite classical MSUD patient, were provided by Dr. Ira K. Brandt (Indiana University, Indianapolis). All fibroblast cell lines were grown as described previously (Chuang and Cox 1988).

cDNA Synthesis and Sequencing

Poly-A$^+$ RNA was isolated from cultured fibroblasts (Chirgwin et al. 1979), and cDNA was synthesized and amplified by the PCR (Saiki et al. 1988). The reaction products were ligated into the blunt-ended restriction site of Bluescript SK II+ plasmid (Stratagene, La Jolla, CA), which were used to transform XL1-Blue cells (Stratagene). Isolated plasmid DNA was sequenced using the dideoxy chain-termination method (Sanger et al. 1977).

Southern Blotting of Genomic DNA

Genomic DNA was prepared and amplified by PCR. The sense primer was complementary to sequence spanning an intron/exon boundary (5′-ATGTCCCCACAGGTGATGGA-3′). The antisense primer was complementary to the sequence in exon 9 of the E1α gene (Dariush et al., in press) (5′-TCTCGGGGTACCTGAGGATGG-3′), except for a single basepair mismatch which generated a *Kpn*I restriction site. The PCR product was electrophoresed on a 1.5% agarose gel and blotted onto Genescreen Plus (Dupont/NEN, Boston). The filter was probed sequentially with end-labeled allele-specific oligonucleotides containing the mutation (5′-GAGCACAACCCACTG-3′) and the normal sequence (5′-GAGCACTACCCACTG-3′) (Zhang et al. 1989).

Results

We have shown previously that the activity of the E1-component is deficient in fibroblasts from PK (Fisher et al. 1989). However, protein contents for E1α and E1β were slightly reduced, and the E2 subunit of the BCKAD complex was present at the normal level. These previous results suggest that a structural mutation may reside in either the E1α or the E1β subunit. To investigate this possible mutation, we amplified and subcloned regions of the E1α cDNA from the proband PK and from the father PaK. Two of the amplified regions, determined by primers 1 and 1′ (bases 1–358) and primers 2 and 4′ (bases 300–1376), are shown in figure 1*A*. The overlapped amplified regions spanned a partial mitochondrial presequence beginning at residue −43, the entire mature sequence, and a portion of 3′ untranslated region of the E1α mRNA (Fisher et al. 1989). Sequencing of the clones carrying either the primer 2 to 4′ region or the primer 1 to 1′ region of the cDNA from PK revealed a consistent T→A nucleotide substitution at base 1307. This changed a tyrosine (TAC) residue at codon 393 of the normal sequence to an asparagine residue (AAC) (fig. 1*B*). The mutation is designated Y393N when single-letter amino acid symbols are used. The T→A nucleotide substitution was also observed in approximately half of the clones harboring the primer 2 to 4′ region of E1α cDNA from the father PaK. No other missense mutations were detected in the amplified E1α cDNA from either PK or PaK.

To confirm the presence of this mutation in the E1α gene, the sequence spanning an intron/exon junction and the 5′ segment of exon 9 was amplified (fig. 2*A*). Ethidium bromide staining showed that the predicted 226-bp fragment was amplified from genomic DNA from all cell lines to be tested (data not shown). Allele-specific oligonucleotide probes of 15 bases were synthesized both to the normal sequence and to the mutant sequence containing the T→A base change. As shown in figure 2*B*, Southern analysis using the mutant probe detected the Mennonite classical MSUD patients (PK and LMK of the K family and MZ, GM 1655, NN, and AF). The mutant probe hybridized with DNA from the obligatory heterozygous parents PaK and MaK and from the putative heterozygous sibling LK (determined by enzyme assays) with reduced intensity. These results indicate the presence of a single copy of the Y393N allele in the heterozygotes. The mutation is absent in controls and in a normal sibling (EK), a non-Mennonite classical MSUD patient (Misk), and a non-Mennonite variant MSUD pa-

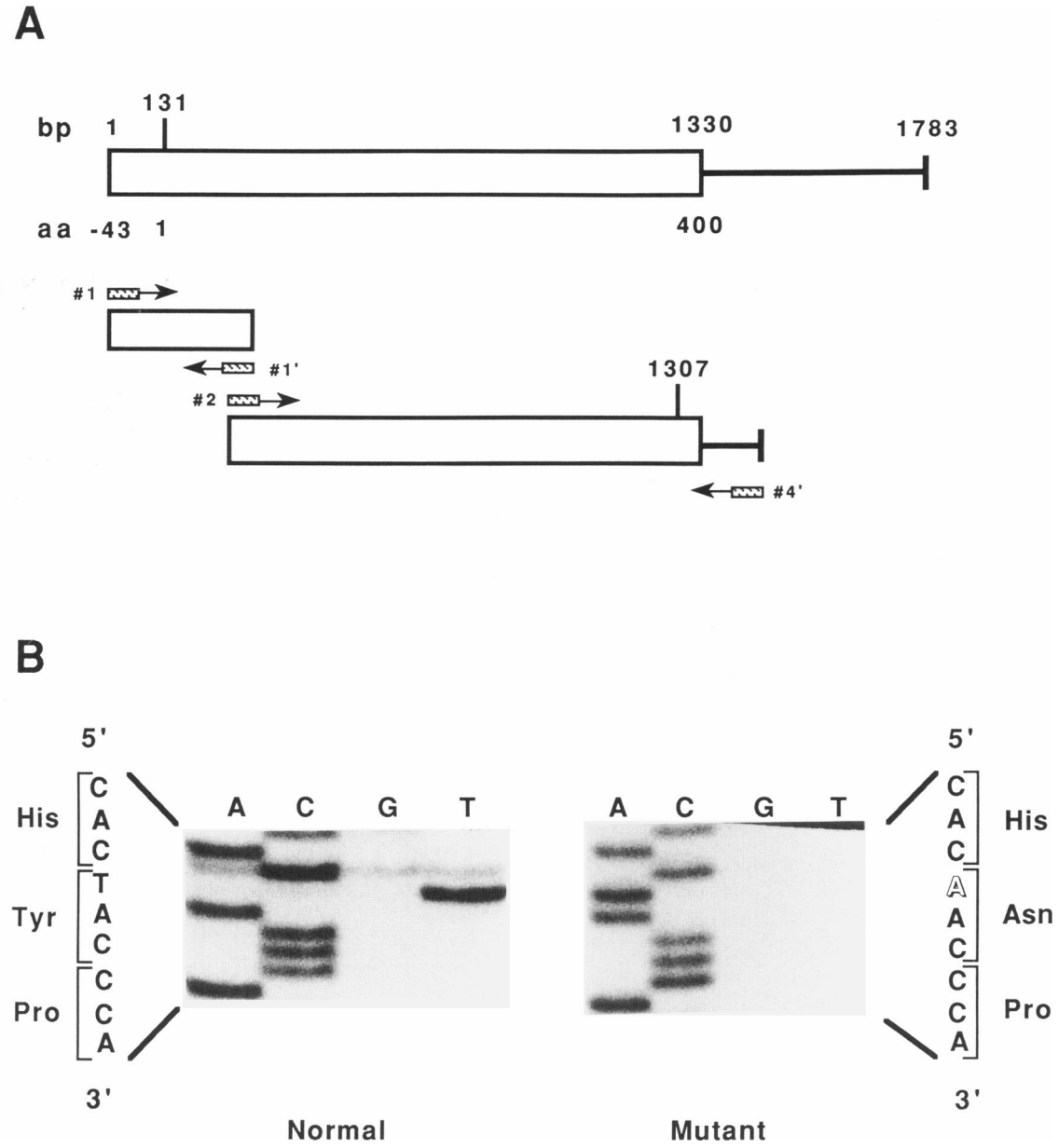

Figure 1 Amplification and sequencing of E1α cDNA from normal and MSUD fibroblasts. *A*, Regions of E1α cDNA amplified by PCR. Open boxes and solid bars represent, respectively, the coding and noncoding sequences of the human E1α cDNA. The entire mature E1α sequence (residues 1–400) and a partial mitochondrial presequence of 43 residues in the cloned cDNA are indicated. The oligonucleotide sense/antisense primers used were 1/1′:5′-CGGGGCGATCGATGCAGCGA-3′/5′-CTCATAGAGGATCCGGTCCA-3′, corresponding to the region of bases 1–300, and 2/4′:5′-TGCTGCAGCTCTACAAGAGCA-3′/5′-TCTCGGGGTACCTGAGGATGG-3′, corresponding to bases 300–1376. The base change at nucleotide 1307 is located in the region spanning the primer 2 to 4′. *B*, Sequences corresponding to residues 392–394 in both normal cDNA and mutant cDNAs from both proband PK, who has classical MSUD, and father PaK. The T→A substitution is shadowed.

tient (M Ko), as indicated by the lack of hybridization signals. The normal oligonucleotide probe shown in figure 2*C* hybridized to DNA from controls and from the normal sibling (EK), the two MSUD patients (M Ko and Misk), and heterozygotes of the K family (PaK, MaK, and LK). The hybridization with both normal and mutant probes in PaK, MaK, and LK confirms the heterozygosity in these subjects. The lack of hybridiza-

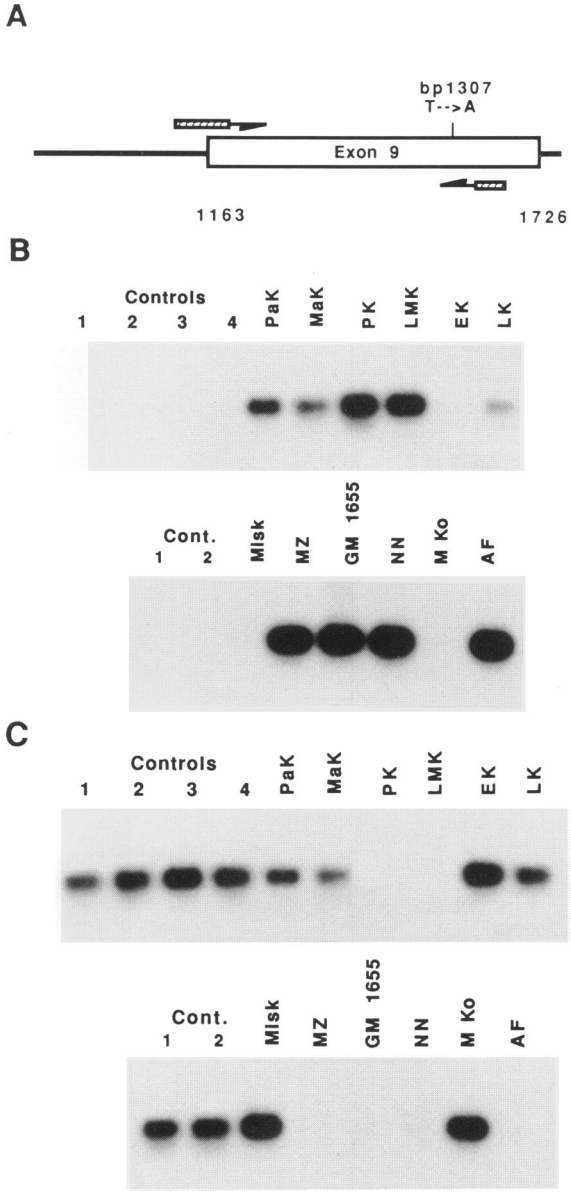

Figure 2 Southern blotting of amplified genomic DNA with normal and mutant allele-specific probes. *A,* Region spanning exon 9 of E1α gene, amplified by PCR. *B,* Amplified products (226 bp) from genomic DNA (2 μg), electrophoresed on 1.5% agarose gels, transferred to Genescreen Plus, and probed with allele-specific oligonucleotide containing T→A mutation (see Material and Methods). The upper set of lanes shows results obtained with members of the Mennonite K family: PaK = father; MaK = mother; PK = proband, who has classical MSUD; LMK = affected sibling; and EK and LK = unaffected siblings. The lower set of lanes shows results obtained with other Mennonite classical MSUD patients (MZ, GM 1655, NN, and AF), a non-Mennonite classical MSUD patient (Misk), and a non-Mennonite variant MSUD patient (M Ko). *C,* Blots identical to those in *B* but probed with normal allele-specific oligonucleotide.

tion of the normal probe to the two affected K family patients (PK and LMK) and to the four other Mennonite classical patients (MZ, GM 1655, NN, and AF) establishes that they are homozygous for the T→A mutation.

Discussion

This communication describes the Y393N mutation that is present in the E1α gene of the BCKAD complex of classical MSUD patients from Philadelphia Mennonites. The mutation is identical to the Y394N reported by Zhang et al. (1989) to be present in one allele from a compound heterozygote with classical MSUD. The discrepancy, in the codon number, between the study of Zhang et al. (1989) and the present report is the result of differences in assigning the amino-terminal residue. Zhang et al. used the rat mature E1α sequence for codon numbering, while in the present study we used the bovine (Hu et al. 1988). Peptide sequencing has shown that the amino-terminal sequence of the rat mature peptide is Phe +1, Pro +2, and Ser +3, and that of the bovine is Phe −1, Ser +1, and Ser +2. The human mature E1α amino-terminal sequence has not been experimentally determined. However, the deduced amino acid sequences around the putative amino-terminal serine of human and bovine E1α are identical (Fisher et al. 1989). Therefore we believe it is more appropriate to number the human codons on the basis of the mature bovine sequence.

In the present study, all six of the Mennonite patients with classical MSUD, including those from the K family, were homozygous for the Y393N mutation, which is unique in the mature E1α subunit. The obligatory heterozygous parents and an unaffected sibling in the K family carry this mutant allele as well as a normal allele. Non-Mennonite classical patients and a non-Mennonite variant patient do not have this mutation. The results show that the Y393N allele segregates within the nuclear Mennonite family and is tightly associated with the MSUD phenotype. Moreover, the Tyr393 residue involved in the missense mutation is conserved among normal E1α sequences of rats (Zhang et al. 1987), cows (Hu et al. 1988), and humans (Zheng et al. 1988; Fisher et al. 1989), a result which is consistent with the important role of this residue in the structure and function of the BCKAD complex. Although the complete mitochondrial targeting presequence has not been cloned, the presence of mature E1α subunit in PK cells (Fisher et al. 1989)

indicates that the presequence functions normally and is removed during the import of the E1α precursor into mitochondria (Hartl et al. 1989).

The expression of the E1α subunit is complex in that the subunit alone has no demonstrable enzymatic activity and requires assembly with E1β to form an active E1 component. The E1 and E3 components bind with the E2 core in mitochondria to constitute the functional BCKAD complex. A possible approach is to transfect, with either the expression vector containing a normal E1α cDNA or a mutant cDNA carrying the Y393N substitution, lymphoblastoid cell lines deficient in the E1α subunit. The ability of the normal and mutant recombinant E1α subunit to restore the BCKAD activity may be studied to assess the functional significance of the Y393N mutation. Alternatively, coexpression of mature E1α and E1β subunits in *Escherichia coli* may be carried out, and the effects that the mutation has on the assembly and function of the E1 component may be studied.

The lymphoblastoid line GM 1655 from a Mennonite classical MSUD patient was shown to be deficient in the E1β subunit, by Western blotting using antiserum against the BCKAD complex (Indo et al. 1987). However, in the present study the quantity of E1α subunit was also reduced in this cell line. This is consistent with our earlier observations with fibroblasts from the index Mennonite classical MSUD patient PK (Fisher et al. 1989). We found that antisera raised against the native BCKAD complex or E1 component has stronger reactivity with the E1α than with the E1β subunit. Therefore, in a Western blot the E1β subunit in GM 1655 cells either may not be detected or may appear to be disproportionally reduced with respect to the E1α subunit. We suggest that the Y393N mutation that produces an altered E1α may affect assembly or stability of the $\alpha_2\beta_2$ structure in E1. Unassembled E1α and E1β are presumably degraded, resulting in reduced steady-state concentrations of both subunits.

The Philadelphia Mennonite classical MSUD patients studied here can trace their ancestry to four men who immigrated from Germany and Switzerland to the United States between the years 1600 and 1750 (Auerbach and DiGeorge 1973). The presence of homozygosity for the Y393N mutation in Philadelphia Mennonite classical MSUD patients suggests the founder effect, in which a member of the founding family must have carried this mutant E1α allele. As the population expanded, the mutant allele reached its current carrier frequency of approximately 1/7 Mennonites, based on the prevalence of 1/176 live births (Marshall and DiGeorge 1981).

The founder effect has often been postulated as the basis for high frequencies of certain genetic diseases associated with particular ethnic groups (Chase 1977). It is interesting that, as one begins to elucidate the molecular basis of the putative founder mutation, allelic heterogeneity is frequently uncovered, particularly in larger populations. For example, the population in Finland has been relatively isolated genetically by geographical, cultural, and linguistic barriers. In a study of gyrate atrophy of the choroid and retina in Finns, at least two missense mutant alleles for ornithine δ-aminotransferase were shown to be independently segregated in the families studied (Mitchell et al. 1989). However, in the Philadelphia Mennonites the population size and the founder number are smaller, and there is relatively stringent consanguinity. It may be that the Y393N allele is the only classical MSUD mutation in this Mennonite population.

In screening 38 additional non-Mennonite MSUD patients, we found three compound heterozygotes for the Y393N mutation (data not shown). As described above, Zhang et al. (1989) previously reported a classical MSUD patient who was also heterozygous for this mutation. The origin of the Y393N allele in these non-Mennonite patients is unknown. There is no CpG dinucleotide involved in the Y393N substitution to suggest that it is a hot spot for mutation (Cooper and Youssoufian 1988). However, on the basis of geographical dispersion and ethnic diversity of the affected individuals, it is possible that the Y393N mutation in non-Mennonites may have arisen independently. Clarification of the origin of this mutation will require examination of polymorphic markers associated with the Y393N mutation in Mennonite and non-Mennonite populations.

The identification of the Y393N mutation in the Mennonite classical MSUD patients will allow prenatal and carrier detection using the DNA-based test. The availability of this method will facilitate an investigation into the frequency and distribution of the mutant allele in this Mennonite population. This approach should be of great importance to the Mennonite community, where the incidence of MSUD is at the highest level.

As an addendum, we would note that, just prior to submission of this communication, Matsuda et al. (1990) in a preliminary publication reported a T→A substitution at codon 394 in the E1α subunit in two

different Mennonite MSUD cell lines (see earlier discussion for explanation of differences in codon numbering). Matsuda et al. also reported that the E1β-subunit sequence was normal in these two cell lines.

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